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Enzymic Degradation of Starch Granules in the Cotyledons of Germinating Peas[1, 2]

Bienvenido O. Juliano[3] and J. E. Varner
AEC Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48823

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Abstract. **Starch, total α- and β-amylase, and phosphorylase levels and the zymogram patterns of these 3 starch-degrading enzymes were determined in the cotyledons of smooth pea (*Pisum sativum* L.) during the first 15 days of germination. Starch is degraded slowly in the first 6 days; during this time, α-amylase is very low, β-amylase is present at a constant level while phosphorylase gradually increases and reaches a peak on the fifth day. Beginning on the sixth day there is a more rapid degradation of starch which coincides with α-amylase production. One phosphorylase band and 2 β-amylase bands are present in the zymogram of the imbibed cotyledon. An additional phosphorylase band and 1 α-amylase band appear during germination. Seeds imbibed in benzyladenine, chloramphenicol, and in cycloheximide show retarded growth and slower starch degradation and enzyme production than the controls. We conclude that α-amylase is the major enzyme involved in the initial degradation of starch into more soluble forms while phosphorylase and β-amylase assist in the further conversion to free sugars.**

The enzymic degradation of starch in plants has been attributed to phosphorylase or α-amylase in the leaves (6, 9) and to α-amylase in starch storage tissues (30) on the basis that starch degradation usually coincides with periods of maximum activity of these enzymes. In an effort to determine more directly and more precisely the relative importance of these enzymes in the breakdown of reserve starch granules, the cotyledons of germinating peas were examined because changes in the levels of amylase (30, 35), starch (1), amylase and starch (3, 29), and phosphorylase (31) have already been much studied in this tissue in different varieties and under different germination conditions. In our study we have followed the levels of starch, dextrin, free sugars, amylases, and phosphorylases of the cotyledons of the cultivar "Early Alaska", a variety of smooth seeds, during germination in the light. The starch granules remaining in the cotyledonary cells after various times of germination were also characterized with respect to their physical and chemical properties to obtain additional information about the degradation which had already occurred.

Materials and Methods

Pea seeds (*Pisum sativum* L.) cv. "Early Alaska" from the Farm Bureau Services, Incorporated, Lansing, Michigan were soaked for 5 to 6 hr in distilled water, dipped for 5 min in 1% sodium hypochlorite, washed with sterile water and planted on moist vermiculite in the diffuse light of the laboratory at 24 to 26°. Pretreatment with various metabolic regulators involved soaking the seeds for 24 hr in aqueous solutions of benzyladenine, chloramphenicol, or cycloheximide.

For carbohydrates determination, 1 or 2 cotyledons were excised from seedlings, rinsed with water, blotted, and soaked in hot 80% (v/v) ethanol and ground in Pyrex glass tissue grinders. The homogenate was centrifuged for 10 min at 20,000*g* and the supernatant fluid collected as the free sugars fraction. The residue was then extracted twice with cold 50% perchloric acid and centrifuged each time for 20 min at 25,000*g*. The combined extract contained the starch and dextrin. For separate dextrin extraction, cold water extraction of the residue from ethanol extraction was performed before that of perchloric acid. Carbohydrate concentration of the extracts was determined colorimetrically by the anthrone-sulfuric acid method of McCready *et al.* (19) with glucose as standard, and results were expressed as mg glucose/2 cotyledons.

Protein extracts were prepared by cutting previously rinsed and blotted cotyledons (2) to small pieces and homogenizing in 2 ml of cold 0.05 M pH 7.0 tris buffer in a Pyrex glass tissue grinder (ice bath). The resulting mixture was centrifuged

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[3] Present address: Chemistry Department, The International Rice Research Institute, Los Banos, Laguna, The Philippines.

for 10 min at 20,000*g* and the supernatant liquid analyzed without further purification for protein content (bovine serum albumin standard) according to Lowry *et al.* (18), for total amylase activity by the method of Chrispeels and Varner (4), for phosphorylase activity as described by Lee (16), and an aliquot used for polyacrylamide gel electrophoresis (27, 28). Modifications of the amylase assay included the use of 1.5 instead of 1.0 ml iodine solution and 4.5 instead of 5.0 ml water due to the presence of iodine-reducing substances in the extract, and the use of 0.05 M pH 4.8 acetate buffer instead of phosphate buffer to suppress phosphorylase activity during the assay. Total (α- and β-) amylase activity was expressed as the decrease in absorbance at 620 mμ per min per 2 cotyledons. The phosphorylase assay was done at room temperature and activity was calculated as μmoles P_i liberated per 5 min per 2 cotyledons.

Polyacrylamide gel electrophoresis followed a modification of the procedure of Scandalios (27) using a 9:1 (v/v) mixture of 0.8 mM citric acid-0.04 M tris pH 8.2 buffer and 0.06 M lithium hydroxide-0.019 M boric acid pH 8.9 buffer in the gel, and the latter buffer for the reservoirs. An 8 % gel was employed to avoid molecular exclusion of enzymes at the origin. The electrophoresis was run for 5 to 6 hr at 4° with a current of 2.5 milliamperes/cm. The gels were then incubated at ambient temperature for at least 4 hr in 0.07 M pH 5.7 phosphate buffer with 0.8 % (w/v) Merck soluble starch as substrate to assay for phosphorylase and amylases, in 0.1 M pH 5.1 citrate buffer containing 0.7 % (w/v) glucose-1-P (dipotassium salt dihydrate, Nutritional Biochemicals Corporation) and 0.8 % (w/v) oyster glycogen as primer to assay for phosphorylase or with 0.8 % (w/v) soluble starch in 0.05 M tris-maleate buffer pH 5.6 for amylases. After this, the gels were stained with dilute I_2-KI solution for a few min to show the zymogram bands, and fixed for several hr in 5 % (v/v) acetic acid in 30 % (v/v) methanol (28).

Crude extracts from dry or imbibed cotyledons, and from cotyledons of 3 to 10 day old seedlings were obtained by homogenizing them in a minimum quantity of tris buffer in a mortar and pestle and centrifuging the mixture 10 min at 20,000*g*. Fractionation of this extract was studied at various ammonium sulfate concentrations of 20, 30, 40, 50, 60, and 70 g/ml extract and zymograms made of both precipitate and supernatant fractions. The seedling extract was fractionated by cold ethanol addition to ethanol concentrations of 40, 45, 50, 55, and 60 % (v/v) followed by electrophoresis of the supernatant fractions. These amylase preparations were identified polarimetrically according to Robyt and French (25) using 0.1 % pea amylose in 0.05 M pH 4.8 acetate buffer as substrate, and a Perkin Elmer Model 141 polarimeter. The action of these extracts on a suspension of Remazolbrilliant blue R-starch (24) (Calbiochem Amylose Azure) in 0.003 % calcium chloride and 0.006 % KH_2PO_4 was determined at room temperature in the presence of toluene vapor.

Starch granules were isolated from cotyledons of 0 day (dry seeds), 5-day, 9-day, and 11-day seedlings by the method of Greenwood and Thomson (13). The excised cotyledons were soaked overnight in 0.01 M mercuric chloride, washed with water, and homogenized in cold 4 % NaCl in a Waring Blendor at medium speed for 3 min. The brei was filtered through cheesecloth, shaken with toluene for at least 6 hr, and the toluene layer with denatured protein siphoned off. Extraction was repeated 3 times or until the toluene layer was no longer turbid. The starch suspension was then centrifuged 15 min at 500*g*, suspended thrice in water, recentrifuged, and air-dried at room temperature. Recovery of the starch ranged from 45 to 70 %. Mean granule size of the samples was obtained by measuring the major axis of the granule from photomicrographs magnified 400-fold.

Starch was defatted with refluxing 85 % (v/v) methanol for 24 hr, air-dried, pre-gelatinized with hot dimethyl sulfoxide, and fractionated as previously described (2). The resulting fractions were analyzed for β-amylolysis limits using Worthington Biochemicals Corporation crystalline β-amylase (13), blue value (absorbance at 680 mμ) (10), intrinsic viscosity in 1 N KOH at 30° using No. 50 or 100 Ubbelohde dilution viscometers (11), and amylopectin mean chain length by periodate oxidation (13).

One-tenth μc of α-D-glucose-U-^{14}C (Schwarz Bioresearch, Incorporated) or sucrose-U-^{14}C (Nuclear Research Chemicals) was applied to the blotted inner surface of a cotyledon of pea seedling. After 20 to 24 hr assimilation time, the cotyledons were washed thoroughly with water and homogenized in hot 80 % ethanol. Starch was isolated according to Hassid and Neufeld (15) without pregelatinization, and the activity of the starting brei and the starch-iodine complex was determined from an aliquot with a Nuclear Chicago Model 1043/1044 planchet counter.

Results

Kinetics of Starch Degradation and of Phosphorylase and Amylase Activity in Cotyledons of Germinating Peas. The degradation of starch in germinating pea cotyledons consists of a slow phase during the first 5 to 6 days of germination in which starch content decreases by 25 % and a fast stage in which the remaining starch is degraded up to 5 times as fast as during the slow stage (Fig. 1). The residual starch after 15 days germination is only 1 % of the original starch content. The original starch content of the cotyledon after correction for dextrin content is 98 mg glucose or 88 mg anhydroglucose/2 cotyledons. This value corresponds to 45 % of the dry weight, based on a dry weight of 194 mg for 2 cotyledons. Reported values for smooth

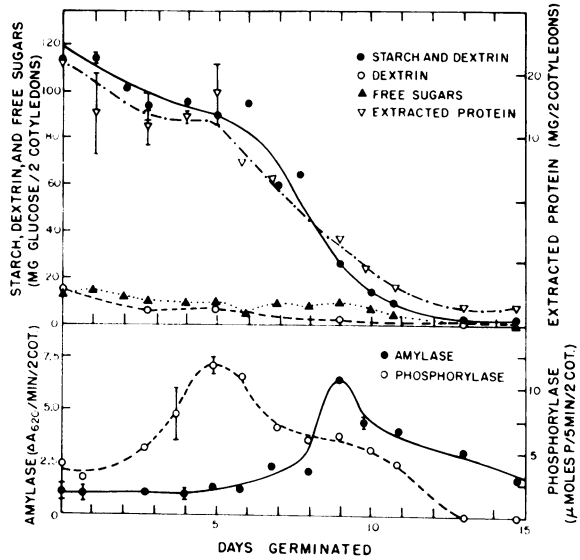

FIG. 1 Changes in the level of starch, dextrin, free sugars, extracted protein, total amylase, and phosphorylase in the germinating pea cotyledons.

pea seeds including testa and axis tissues ranged from 42 to 45 % (13, 19). The other carbohydrate fractions, dextrin and free sugars, do not show a peak but decrease gradually from the 0 day value. The starch degradation of this cultivar occurs earlier and faster than that reported for other varieties (1, 3, 29).

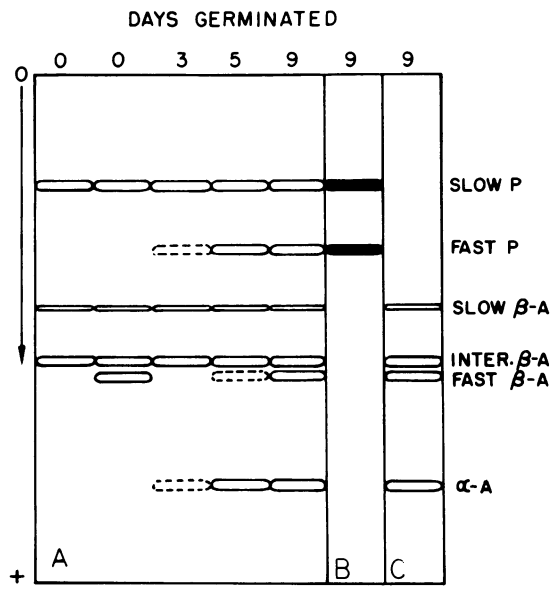

FIG. 2. Schematic zymogram of germinating pea cotyledon α- and β-amylases (A) and phosphorylases (P). O′ denotes 0-day extract stored 5 days at 4°. Incubation medium: 0.8 % (w/v) starch in phosphate buffer (A); 0.7 % glucose-1-P and 0.8 % (w/v) glycogen as primer in citrate buffer (B); or 0.8 % (w/v) starch in tris-maleate buffer (C). Stain was iodine.

The level of protein in the cotyledon extracted in tris buffer decreases slowly during the first 5 days of germination. Thereafter it decreases rapidly and is only 7 % of the original content by the fifteenth day (Fig. 1). In some cases, the protein content of the fifth day extract was higher than that of the third day. This protein fraction represents 50 % of the crude protein content in the imbibed seed and probably contains some globulin as well as the albumins (5). Similar decreases in protein level have been reported by others (1, 3, 5). Amylase level in the tris extract remains low and constant during the first 4 days of germination and increases to a maximum (6-fold) level by the ninth day. This period coincides with the rapid decrease in extracted protein. Although this colorimetric assay has been used predominantly for α-amylase activity, β-amylase also reduces the iodine-blue color of starch at a slower rate than α-amylase. Similar increases in activity of amylase during this period have been reported for this cultivar grown in the light (33) and in the dark (35).

Phosphorylase activity increases, to a 3-fold level, by the fifth day of germination. Swain and Dekker (31), using the same cultivar grown in the dark, noted an increase in phosphorylase activity of more than 13-fold between the first and fourth day of germination.

The Pattern of Starch-degrading Enzymes in the Pea Cotyledons. The zymogram pattern of the starch degrading enzymes shows a maximum of 6 bands (Fig. 2). The 2 slowest bands and the fastest band were colorless in gels incubated in starch in phosphate buffer and stained with I_2, but only the fastest band showed activity with the same substrate in tris-maleate buffer. The 3 middle bands were pink in color after incubation in starch and staining with I_2 and were present in starch-incubated gels regardless of the buffer used. The 2 slow bands were the only ones which synthesized blue-iodine-staining bands from glycogen primer and glucose-1-P. These results are consistent with the identification of these 2 slowest bands as phosphorylases. They can degrade starch only in the presence of P_i; this explains their absence in the gel incubated in tris-maleate buffer. Longer incubation time was required when glycogen was deleted from the citrate buffer containing glucose-1-P. Although this may mean that the phosphorylases do not require a primer as does the maize phosphorylase II of Tsai and Nelson (32), a more likely explanation is that the phosphorylase has some primer complexed with it. Such phosphorylase-glycogen complexes were observed in gels in which 0.2 % glycogen was added to the gel before polymerization. The portion incubated in glucose-1-P showed, on iodine staining, 3 bands of which only the slowest one showed activity with starch in phosphate buffer as substrate. No additional phosphorylase band appeared with the addition of 0.1 % AMP to the incubation medium indicating

the absence in cotyledons of germinating pea of phosphorylases which require this coenzyme (7).

The pink color reaction of the bands with intermediate migration rates with starch and amylopectin indicate incomplete hydrolysis of the substrate. These bands were absent in gels incubated in glycogen plus glucose-1-P. Fractionation with ammonium sulfate of extracts of imbibed cotyledons showed that most of the activity of this enzyme was precipitated with 20 g ammonium sulfate/100 ml extract as evident from the zymograms. Polarimetric study of the action of this enzyme preparation on amylose in acetate buffer gave an upward mutarotation of the hydrolysate on sodium carbonate addition. This indicates that the sugar hydrolysate had the β-configuration (25). This extract has no activity toward Remazolbrilliant Blue R-starch, but starch can be hydrolyzed by α-amylase but not by hydrolyzes amylopectin. Remazolbrilliant Blue R-β-amylase (24). Heating for 15 min at 70° inactivated this enzyme, which is typical for β-amylase (8). These properties formed the bases for designating these enzyme bands as β-amylase. β-Amylase has been shown to be present in the mature pea cotyledon by Swain and Dekker (31) but their β-amylase from axis tissue was more soluble in ammonium sulfate than this cotyledon enzyme. In fact, the cotyledon fraction precipitating between the salt concentrations of 23 to 53 g/100 ml had no β-amylase bands in its zymogram. Although Q (branching) enzyme also has the same color reaction with starch as β-amylase, the former has little activity on amylopectin (14), whereas β-amylase is active on this substrate.

The fastest migrating band in the zymogram from pea cotyledons was more soluble in ethanol than β-amylase since no β-amylase bands were noted in the 50 % ethanol supernatant liquid upon assay. This enzyme was stable to heating for 15 min at 70° (8) at pH 5.3 in the presence of Ca^{2+} and hydrolysates from the action of this enzyme on amylose showed a downward mutarotation. These are characteristics of α-amylase. The enzyme slowly hydrolyzed Remazolbrilliant Blue R-starch into a deep-blue solution. Only α-amylase can liberate a blue dextrin-dye hydrolysate from this starch derivative. The blue dye is covalently linked to the primary hydroxyl group of starch and this dye substituent is a barrier to β-amylase and phosphorylase action on starch (17, 24). These reactions permit identification of this band as α-amylase. Thus, of the 6 starch-degrading enzymes of cotyledons of germinating smooth pea, 2 are phosphorylases, 3 are β-amylases and 1 an α-amylase.

Changes of the Enzyme Pattern During Germination. In the 5-hr imbibed seed, only the slow phosphorylase and the β-amylase bands were detected. The fast phosphorylase and the α-amylase bands are detectable by the third day of germination. The fast β-amylase band appeared about the ninth day of germination but it also appeared during 0° storage of an extract from imbibed seeds (Fig. 2). Thus, the fast phosphorylase and the single α-amylase bands are presumably the only 2 new enzymes in the cotyledons which are produced during germination. In gels with 0.2 % amylopectin, a blue-staining band was observed in germinated seedling extracts with a mobility intermediate between the phosphorylases and β-amylases. This probably is the R or debranching enzyme which can synthesize amylose from amylopectin and which has been detected electrophoretically in maize endosperm (20).

Properties of Starch in the Pea Cotyledons. Some properties of the starch granules isolated from the mature seed and germinating pea cotyledons are shown in table I. Most of the granules had cracks along their major axis. The values obtained for the seed (0-day) starch were comparable to previously reported values for smooth pea starch for granule size of 30 to 40 μ (13, 23); β-amylolysis limits of 80 and 81 % (13): amylopectin intrinsic viscosity

Table I. *Some Properties of Cotyledon Starch Granules of Germinating Pea*

Property	Days germinated		
	0	5	11
Starch			
Mean granule size, μ	28.9 ± 2.2	25.0 ± 1.2	20.8 ± 1.4
Blue value, Abs. at 680 mμ	0.351	0.368	0.366
Amylopectin			
Intrinsic viscosity, ml/g	182	112	95
Mean chain length, anhydro-glucose units	26.6 ± 0.9	26.5 ± 0.6	21.8 ± 0.0
β-Amylolysis limit, %	57	53	47
Apparent inner chain length,[1] anhydro-glucose units	9	10	9
Amylose			
Blue value, Abs. at 680 mμ	1.02	0.92	1.05
Intrinsic viscosity, ml/g	170	163	151
β-Amylolysis limit, %	84	89	96

[1] Calculated from chain length—[(chain length × β-limit) + 2.5]; results were corrected to nearest whole number (13).

of 140 to 157 ml/g (13, 23); of 57 and 58 % (13); β-limit and mean chain length of 26 to 27 anhydroglucose units (13, 23).

The mean granule size of the starch decreases during germination (table I). A slight increase in the blue value during germination indicated that the amylopectin fraction was slightly more susceptible to enzymatic solubilization in the starch granule than was amylose.

The molecular size of both starch fractions as indexed by intrinsic viscosity decreased during germination. However, the degradation of the amylopectin fraction was much more extensive by the fifth day, especially considering that a branched polymer like amylopectin changes less in viscosity per unit change in molecular size than its linear analogue, amylose. Since its chain length remained the same up to this time, this drop in viscosity must be due to scission of inner chain α-1,4-glucosidic linkages by α-amylase. The amylopectin of the residual starch of the eleventh day sample had a shorter outer chain length than the earlier samples, probably because of the action of β-amylase and of phosphorylase. Such increases in β-limit values can be explained only by α-amylolysis of inner α-1,4-glucosidic bonds, which exposes more non-reducing ends to β-amylase action. These results indicate that α-amylase is the major enzyme involved in degrading starch granules. Similar but less extensive degradation of the starch fractions were observed during the malting of barley (12).

Starch Turnover in Cotyledons of Germinating Peas. Labeling of the cotyledon on the second to the seventh day of germination with α-D-glucose-U-^{14}C for 20 to 25 hr resulted in the incorporation of 5 to 12 % of the radioactivity taken up (7000–24,000 cpm) into the starch fraction. Practically the same results were obtained with sucrose-U-^{14}C. These results indicate that turnover of residual starch molecules occurred even during the rapid phase of starch degradation.

Effects of Benzyladenine and Protein Synthesis Inhibitors on Starch and Amylase of Pea Cotyledons. The effect of presoaking the seed in metabolic inhibitors on starch and amylase levels of the cotyledon during germination is shown in Fig. 3. Zymograms of these extracts showed simultaneous appearances of the α-amylase and phosphorylase (fast) bands. Growth, and starch and enzyme levels of the third-day samples were the same as in the control. However, later samples showed that pretreatment with 15 and 30 μg/ml chloramphenicol retarded growth, starch degradation, and enzyme production. Cycloheximide at a concentration of 2 μg/ml had no effect on germination.

Benzyladenine pretreatment caused thickening and stunting of the axis and as many as 4 lateral shoots had grown at the cotyledonary node by the seventh day of germination. The starch and α-amylase data for the samples pretreated with benzyladenine were

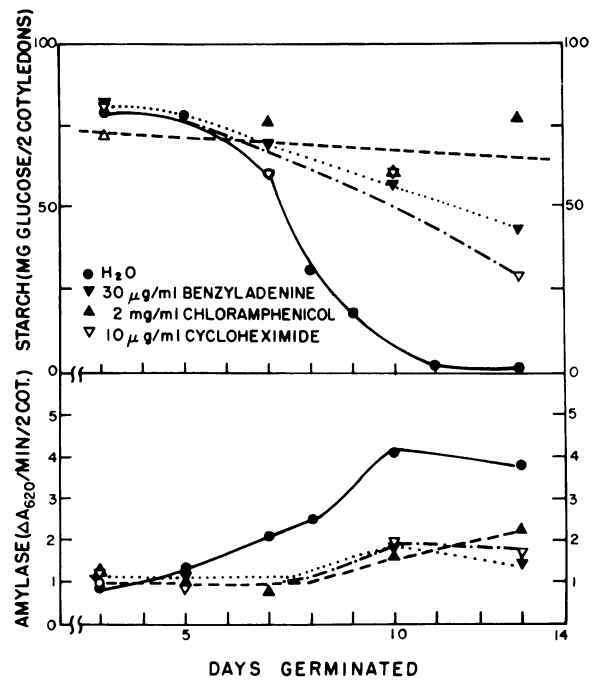

FIG. 3. Changes in the starch and total amylase contents of germinating pea cotyledons pretreated with various metabolic inhibitors

similar to but less dramatic than those reported for 2 other pea varieties (29).

Chloramphenicol pretreatment produced chlorotic seedlings. This chemical has previously been reported to inhibit chloroplast synthesis (21). The samples pretreated with 5 mg/ml of this reagent all died within 9 days of germination, and only a few of those treated with 2 mg/ml were able to continue growth and produce green shoots by the thirteenth day. Growth was very slow, in agreement with previous observations on samples grown in the dark (35). Cycloheximide at a concentration of 10 μg/ml greatly retarded seedling growth, starch degradation, and α-amylase production (Fig. 3).

Discussion

During the first 5 or 6 days of germination there is a slow disappearance of starch from the cotyledons. The rapid disappearance of starch which begins on the sixth day coincides with the appearance of an α-amylase band and a second phosphorylase band on the gel electrophoresis zymograms. We conclude from these results that even though there is β-amylase and phosphorylase activity (2 or 3 bands and 1 band, respectively, on the zymograms) in the dry cotyledons and throughout the period of growth studied, these enzymes alone cannot bring about a rapid degradation of starch. α-Amylase, and perhaps the second phosphorylase, is required for rapid degradation of starch. The third (fast)

β-amylase band which appears during germination can also be produced by storing the extract of dry cotyledons for a few days at 0°. We conclude that the fast β-amylase band is present in a latent form in the seed and becomes freed during germination. A similar activation has been observed in wheat. β-Amylases which are attached by disulfide bonds to reserve proteins are freed by reduction during germination and can be freed *in vitro* by treatment of homogenates with β-mercaptoethanol (26). The constant low total amylase activity during the first few days must be due to β-amylase, which also reduces the starch-iodine color at 620 mμ.

A similar, low activity of β-amylase in pea cotyledons has been reported by Swain and Dekker (31) throughout 8 to 10 days growth in the dark. Matile (22) reported β-amylase activity of protein bodies from pea cotyledons isolated 1 and 3 days after germination.

Not only does the rapid increase of α-amylase activity coincide with the faster rate of starch degradation but also the drop in intrinsic viscosity of the amylopectin with no apparent change in its outer chain length is consistent only with α-amylolysis of its inner chains to produce lower molecular weight fragments with the same degree of branching. Phosphorylase and β-amylase can only act stepwise on the outer chains of amylopectin (13, 17). The drop in viscosity of amylose during this period of rapid starch degradation also cannot be due to β-amylase and/or phosphorylase action alone as otherwise the β-limit should decrease rather than increase. These results indicate that α-amylase is the major enzyme involved in the initial degradation of starch into the more soluble forms, which other enzymes such as β-amylase and phosphorylase assist in converting into free sugars. This latter conversion of degraded starch into dextrin and ultimately into free sugars, and subsequent translocation from the cotyledon must be very efficient since no appreciable accumulation of dextrin or free sugars was observed. Presumably, the low α-amylase level is the reason for the slow rate of starch degradation during the first 4 days of germination. The incorporation of α-D-glucose-U-^{14}C into the starch of 7-day seedling cotyledons showed turnover of the starch even during its rapid degradation. *In vitro* studies have shown that only α-amylase can react on starch granules (34). However, the periods of activity of phosphorylase and amylases overlapped in this system, and complete separation of the periods of activity of these enzymes by blocking the production of either enzyme is required to prove unequivocably that α-amylase is the major enzyme for initial degradation of the starch granule. The leaves of 79 species of plants—all those studied—were recently reported to have α-amylase activity (9).

Attempts were made to inhibit selectively the production of either phosphorylase or α-amylase in the cotyledon of germinating pea by the use of metabolic inhibitors. However, in all treatments their sequence of production remained the same, although their rates of production were reduced. Germination in the dark did not inhibit the production of phosphorylase or α-amylase. Presumably, the sequence of production of these 2 enzymes in the dark is the same as in the light since Swain and Dekker (31) found peak phosphorylase activity at about the eighth day, whereas Young and Varner (36) reported the specific activity of α-amylase as still increasing 8 days after germination.

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